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<b>(21) International Application Number:</b> PCT/AU87/00172 <b>(22) International Filing Date:</b> 12 June 1987 (12.06.87)  <b>(31) Priority Application Number:</b> PH 6385 <b>(32) Priority Date:</b> 12 June 1986 (12.06.86) <b>(33) Priority Country:</b> AU  <b>(71) Applicants (for all designated States except US):</b> THE UNIVERSITY OF MELBOURNE [AU/AU]; Grat-tan Street, Parkville, VIC 3052 (AU). VICTORIAN DAIRY INDUSTRY AUTHORITY [AU/AU]; Dom-ville Avenue, Hawthorn, VIC 3122 (AU).  <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only) :</b> REYNOLDS, Eric, Charles [AU/AU]; 14 Kelvinside Street, North Bal-wyn, VIC 3103 (AU).	<b>(74) Agent:</b> SANDERCOCK, SMITH & BEADLE; 207 Riversdale Road, Hawthorn, VIC 3122 (AU).  <b>(81) Designated States:</b> AT (European patent), AU, BB, BE (European patent), BG, BJ (OAPI patent), BR, CF (OAPI patent), CG (OAPI patent), CH (European pa-tent), CM (OAPI patent), DE (European patent), DK, FI, FR (European patent), GA (OAPI patent), GB (European patent), HU, IT (European patent), JP, KP, KR, LK, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL (Euro-pean patent), NO, RO, SD, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent), US.  <b>Published</b> <i>With international search report.</i>	
<b>(54) Title:</b> PHOSPHOPEPTIDES  <b>(57) Abstract</b>  A phosphopeptide or a salt thereof the phosphopeptide having from 5 to 30 amino acids including the sequence A-B-C-D-E where A, B, C, D and E are independently phosphoserine, phosphothreonine, phosphotyrosine, phosphohistidine, glutamate and aspartate and compositions particularly compositions to teeth including same.		

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1 TITLE: PHOSPHOPEPTIDES

2 This invention relates to phosphopeptides and  
3 compositions containing same.

4 This invention also relates to caries and gingivitis  
5 inhibition.

6 The present invention provides a phosphopeptide or a  
7 salt thereof, the phosphopeptide having from 5 to 30 amino  
8 acids including the sequence

9 A-B-C-D-E

10 where A,B,C,D and E are independently phosphoserine,  
11 phosphothreonine, phosphotyrosine, phosphohistidine,  
12 glutamate and aspartate.

13 Preferred phosphopeptides are those wherein A,B and C  
14 are independently phosphoserine, phosphothreonine,  
15 phosphotyrosine and phosphohistidine and D and E are  
16 independently phosphoserine, phosphothreonine, glutamate and  
17 aspartate.

18 Particularly preferred phosphopeptides are those where  
19 A,B and C are phosphoserine and D and E are glutamate.

20 The phosphopeptide is preferably in substantially pure  
21 form.

22 The phosphopeptides of the present invention or their  
23 salts may have utility in the treatment or inhibition of (i)  
24 dental diseases such as caries, gingivitis and periodontal  
25 disease, (ii) rarefying bone diseases such as osteoporosis  
26 and osteomalacia and (iii) diseases relating to  
27 malabsorption of minerals.

28 Accordingly, the present invention provides a  
29 composition comprising a peptide or a salt thereof in  
30 accordance with this invention and a physiologically  
31 acceptable diluent.

32 The composition may be in the form of a pharmaceutical  
33 composition.

34 The composition may be orally ingestible.

35 A mixture of phosphopeptides and /or their salts may be  
36 used in the composition. In this instance it is preferred  
37 that those containing the sequence A-B-C-D-E above  
38 predominate.

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1       The phosphopeptide or mixture of phosphopeptides is  
2 preferably substantially pure at least to the extent of not  
3 containing unpalatable impurities.

4       The following phosphopeptides have been found to be  
5 useful in the compositions of the present invention:-

- 6 T1.Glu-Met-Glu-Ala-Glu-Pse-Ile-Pse-Pse-Pse-Glu-Glu-Ile-Val-  
7 Pro-Asn-Pse-Val-Glu-Gln-Lys,  
8 T2.Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu-Pse-  
9 Leu-Pse-Pse-Pse-Glu-Glu-Ser-Ile-Thr-Arg,  
10 T3.Asn-Thr-Met-Glu-His-Val-Pse-Pse-Pse-Glu-Glu-Ser-Ile-Ile-  
11 Pse-Gln-Glu-Thr-Tyr-Lys,  
12 T4.Asn-Ala-Asn-Glu-Glu-Glu-Tyr-Ser-Ile-Gly-Pse-Pse-Pse-Glu-  
13 Glu-Pse-Ala-Glu-Val-Ala-Thr-Glu-Glu-Val-Lys, and  
14 T5.Glu-Gln-Leu-Pse-Pth-Pse-Glu-Glu-Asn-Ser-Lys.

15       The amino acid symbols are as follows : Pse-  
16 phosphoserine, Ser-Serine, Pth-phosphothreonine, Thr-  
17 threonine, Glu-glutamate, Asp-aspartate, Ala-alanine, Asn-  
18 asparagine, Gln-glutamine, Gly-glycine, Arg-arginine, His-  
19 histidine, Ile-isoleucine, Leu-leucine, Lys-lysine, Met-  
20 methionine, Pro-proline, Tyr-tyrosine, Val-valine.

21       The phosphopeptide may be made synthetically by  
22 chemical synthesis or genetic engineering or can be  
23 extracted from naturally occurring material.

24       Because of cost considerations it is currently more  
25 economic to extract the phosphopeptide from casein and in  
26 particular from alpha-s casein or beta-casein. Phosvitin  
27 may also be used as a source of the peptide. Further,  
28 phosphoproteins in cereals, nuts and vegetables particularly  
29 in bran husks or sheaths may be used to produce the peptide  
30 above. In particular, rice, wheat, oat, barley or rye  
31 brans. Soybean and meat contain phosphoproteins which may  
32 be of use in obtaining the peptide above.

33       Casein and in particular alpha-s casein or beta-casein  
34 or salts thereof such as sodium caseinate contain  
35 polypeptides which can be cleaved to simpler peptides.  
36 Such cleavage may be effected by digestion, such digestion  
37 may be chemical or proteolytic.

38       It is presently preferred to digest casein with one of

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1 trypsin, pepsin, chymotrypsin, papain, thermolysin or  
2 pronase. Of these, trypsin is preferred.

3 The digested casein can be fractionated into peptides  
4 including the sequence A-B-C-D-E and other peptides. The  
5 presence of said other peptides is not deleterious to  
6 efficacy, however, certain of said other peptides have  
7 objectionable taste and accordingly if any of said other  
8 peptides are to be included it is preferable to remove those  
9 having objectionable taste. In general, those of said  
10 other peptides having objectionable taste seem to be  
11 hydrophobic.

12 The following peptides have been found to have  
13 objectionable taste:-

- 14 1. Glu-Val-Leu-Asn
- 15 2. Asn-Glu-Asn-Leu-Leu
- 16 3. Ala-Pro-Phe-Pro-Gln-Val-Phe-Gly
- 17 4. Leu-Arg-Phe
- 18 5. Phe-Phe-Val-Ala-Pro-Phe-Pro-Gln-Val-Phe-Gly-Lys
- 19 6. Leu-Arg-Leu
- 20 7. Phe-Tyr-Pro-Glu-Leu-Phe

21 (Glu-glutamate; Val-valine; Leu-leucine; Asn-asparagine;  
22 Ala-alanine; Pro-proline; Phe-phenylalanine; Gln-glutamine;  
23 Gly-glycine; Arg-Arginine; Lys-lysine; Tyr-tyrosine.)

24 Preferably the peptide is one exhibiting a reduction in  
25 hydroxy apatite dissolution rate of at least 15% under the  
26 test conditions defined herein.

27 Preferably the peptide is one exhibiting a reduction  
28 in hydroxy apatite dissolution rate of at least 26% under  
29 the test conditions defined herein.

30 Preferably, the peptide is one exhibiting a reduction  
31 in hydroxy apatite dissolution rate of at least 30% under  
32 the test conditions defined herein.

33 Preferably, the peptide is one exhibiting a reduction  
34 in hydroxy apatite dissolution rate of at least 32% under  
35 the test conditions defined herein.

36 Preferably, the peptide is present as 0.01 to 10% by  
37 weight.

38 Preferably, the peptide is present as 0.01 to 5% by

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1 trypsin, pepsin, chymotrypsin, papain, thermolysin or  
2 pronase. Of these, trypsin is preferred.

3 The digested casein can be fractionated into peptides  
4 including the sequence A-B-C-D-E and other peptides. The  
5 presence of said other peptides is not deleterious to  
6 efficacy, however, certain of said other peptides have  
7 objectionable taste and accordingly if any of said other  
8 peptides are to be included it is preferable to remove those  
9 having objectionable taste. In general, those of said  
10 other peptides having objectionable taste seem to be  
11 hydrophobic.

12 The following peptides have been found to have  
13 objectionable taste:-

- 14 1. Glu-Val-Leu-Asn
- 15 2. Asn-Glu-Asn-Leu-Leu
- 16 3. Ala-Pro-Phe-Pro-Gln-Val-Phe-Gly
- 17 4. Leu-Arg-Phe
- 18 5. Phe-Phe-Val-Ala-Pro-Phe-Pro-Gln-Val-Phe-Gly-Lys
- 19 6. Leu-Arg-Leu
- 20 7. Phe-Tyr-Pro-Glu-Leu-Phe

21 (Glu-glutamate; Val-valine; Leu-leucine; Asn-asparagine;  
22 Ala-alanine; Pro-proline; Phe-phenylalanine; Gln-glutamine;  
23 Gly-glycine; Arg-Arginine; Lys-lysine; Tyr-tyrosine.)

24 Preferably the peptide is one exhibiting a reduction in  
25 hydroxy apatite dissolution rate of at least 15% under the  
26 test conditions defined herein.

27 Preferably the peptide is one exhibiting a reduction  
28 in hydroxy apatite dissolution rate of at least 26% under  
29 the test conditions defined herein.

30 Preferably, the peptide is one exhibiting a reduction  
31 in hydroxy apatite dissolution rate of at least 30% under  
32 the test conditions defined herein.

33 Preferably, the peptide is one exhibiting a reduction  
34 in hydroxy apatite dissolution rate of at least 32% under  
35 the test conditions defined herein.

36 Preferably, the peptide is present as 0.01 to 10% by  
37 weight.

38 Preferably, the peptide is present as 0.01 to 5% by

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1 weight.

2 Preferably, the peptide is present as 0.01 to 2% by  
3 weight.

4 The composition of this invention may be in the form of  
5 a comestible such as foodstuff or confectionary, dentifrice,  
6 tablet or comprise a pharmacologically acceptable vehicle or  
7 solution of suspension for topical application to the teeth  
8 or gingival tissues or a mouthwash. Other modes of  
9 administering the peptide would be acceptable if  
10 physiologically or pharmacologically acceptable.

11 Of particular interest as compositions are chewing gum,  
12 breakfast foods, ice-cream and other frozen confectionery,  
13 confectionery, sweets and cakes as these are all known as  
14 caries problem materials. Similar considerations apply to  
15 other potentially cariogenic food components.

16 Also of particular interest are dentifrices,  
17 mouthwashes and preparations for topical application to  
18 teeth and gingival tissue and enteric capsules for the  
19 treatment of bone disorders and mineral malabsorption.

20 Also of interest is the use of compositions in  
21 accordance with this invention in respect of dental  
22 treatment of cavities. In this last respect, there appears  
23 to be evidence of remineralization of incipient lesions  
24 which are considered to be a pre-cavity condition. However,  
25 there is also evidence to indicate that application of  
26 compositions in accordance with this invention to the  
27 surfaces of actual cavities and to surfaces of teeth  
28 produced by removal of decay material from actual cavities  
29 or by fracture is beneficial.

30 Since a topical application of a composition in  
31 accordance with this invention which is an aqueous solution  
32 to surfaces of actual cavities or surfaces of teeth produced  
33 by removal of decay material from actual cavities or by  
34 fracture is unlikely to have long term effect, we have  
35 further sought to provide compositions which might have the  
36 desired long term effect.

37 Accordingly, the present invention also provides a  
38 composition in accordance with this invention and adapted to

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1 remain in contact with a tooth surface over a prolonged  
2 period. The invention also provides methods and means for  
3 maintaining compositions in accordance with this invention  
4 in contact with a tooth surface over a prolonged period.

5 In this last respect a prolonged period should be  
6 interpreted in accordance with the effect desired and the  
7 time taken to achieve sufficient of that effect to be of  
8 value. However, in some instances that prolonged period may  
9 be as short as one day but is more preferably a period of  
10 weeks or months.

11 In one instance a tooth cavity is coated with a  
12 composition in accordance with this invention and the cavity  
13 is closed to restrict escape of the composition. Such  
14 closure may be effected by capping or use of dental cavity  
15 filling compositions.

16 In another instance the composition is so formulated as  
17 to be adapted to remain in place for a prolonged period. In  
18 this instance the composition of the invention may form part  
19 of a dental filling composition.

20 Accordingly, the present invention also provides a  
21 dental filling composition comprising a phosphopeptide of  
22 formula A-B-C-D-E as defined above and a carrier therefor  
23 adapted to adhere the composition to a tooth surface.

24 Such a dental filling composition may contain dental  
25 filling materials known per se including amalgams and  
26 settable polymers.

27 Of particular interest are dental filling compositions  
28 which contain calcium. The calcium is desirably in the form  
29 of calcium phosphate or hydroxyapatite.

30 The phosphopeptides for use in the invention can be  
31 extracted in a number of ways but the use of a fractionation  
32 technique is generally preferred.

33 The phosphopeptides can be extracted by fractionation  
34 based on molecular size or charge characteristics. Due to  
35 the unique negative charge density and divalent metal ion  
36 sequestering ability of the peptides conferred by the active  
37 sequence A-B-C-D-E as defined, the preferred fractionation  
38 procedure is anion exchange chromatography or selective

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1 precipitation or a combination of both.

2 The following procedure illustrates one mode of  
3 extraction.

4 Extraction Procedure I.

5 An example of the phosphopeptides are those produced by  
6 a tryptic digestion of bovine milk casein. The digestion  
7 of whole sodium caseinate or fractions (alpha-S or beta)  
8 produced by selective precipitation (Zittle, C.A. and Custer  
9 J.H.; J. Dairy Sci 48L 1183-1189, 1963) is carried out using  
10 a protein: trypsin ratio of 50:1 in 20 mM Tris HCl pH 8.0,  
11 2.5mM NaCl at 37°C for 1h. The peptides were fractionated  
12 using a Pharmacia FPLC system with a Mono Q HR 5/5 column  
13 and eluted with a NaCl gradient; Buffer A 20mM Tris HCl pH  
14 8.0, 2.5mM NaCl; Buffer B 20 mM Tris HCl pH 8.0, 500mM  
15 NaCl, gradient 0-100% Buffer B/30 min; flow rate 1ml/min.  
16 Fractions were washed and concentrated using an Amicon  
17 Ultrafiltration Cell with a UM05 filter. The peptides were  
18 identified using a Water Associates PICO-TAG amino acid  
19 analysis system using phenylisothiocyanate amino acid  
20 derivatisation. Phosphate was measured by the method of  
21 Itaya and Ui (Clin. Chim. Acta. 14:361-366, 1960). The  
22 peptides were sequenced (after the removal of phosphate by  
23 alkaline phosphatase) using manual Edman degradation and the  
24 resulting PTH-amino acids identified using reverse phase  
25 HPLC on a Zorbax ODS column 25x0.46 cm (DuPont).

26 The following phosphopeptides were individually  
27 obtained from a tryptic digestion of sodium caseinate using  
28 the above procedure.

29 T1.Glu-Met-Glu-Ala-Glu-Pse-Ile-Pse-Pse-Pse-Glu-Glu-Ile-Val-  
30 Pro-Asn-Pse-Val-Glu-Gln-Lys.

31 T2.Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu-Pse-  
32 Leu-Pse-Pse-Pse-Glu-Glu-Ser-Ile-Thr-Arg.

33 T3.Asn-Thr-Met-Glu-His-Val-Pse-Pse-Pse-Glu-Glu-Ser-Ile-Ile-  
34 Pse-Gln-Glu-Thr-Tyr-Lys.

35 T4.Asn-Ala-Asn-Glu-Glu-Glu-Tyr-Ser-Ile-Gly-Pse-Pse-Pse-Glu-  
36 Glu-Pse-Ala-Glu-Val-Ala-Thr-Glu-Glu-Val-Lys.

37 T5.Glu-Gln-Leu-Pse-Pth-Pse-Glu-Glu-Asn-Ser-Lys.

38 In addition the following peptides were also obtained:



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- 1 T6.Asp-Ile-Gly-Pse-Glu-Pse-Thr-Glu-Asp-Gln-Ala-Met-Glu-Asp-  
2 Ile-Lys.  
3 T7.Val-Pro-Gln-Leu-Gln-Ile-Val-Pro-Asn-Pse-Ala-Glu-Glu-Arg.  
4 T8.Thr-Val-Asp-Met-Glu-Pse-Thr-Glu-Val-Phe-Thr-Lys.  
5 T9.Leu-Pth-Glu-Glu-Lys.

6 The peptides T1, T6 and T7 were also obtained from a  
7 TPCK-tryptic digest of  $\alpha_{s1}$ -caseinate (comprising  $\alpha_{s1}$   
8 and  $\alpha_{s6}$ ). Peptide T2 was also obtained from a TPCK-  
9 tryptic digest of beta-caseinate. Peptides T3, T4, T5, T8  
10 and T9 were also obtained from a TPCK-tryptic digest of  
11  $\alpha_{s2}$ -caseinate (comprising  $\alpha_{s2}$ ,  $\alpha_{s3}$ ,  $\alpha_{s4}$  and  
12  $\alpha_{s6}$ ). The amino acid symbols are as follows: Pse-  
13 phosphoserine, Ser- serine, Pth-phosphothreonine, Thr-  
14 threonine, Glu- Glutamate, Asp- aspartate, Ala- alanine,  
15 Asn- asparagine, Gln- glutamine, Gly- glycine, Arg- arginine,  
16 His- histidine, Ile- isoleucine, Leu- leucine, Lys- lysine,  
17 Met - methionine, Pro- proline, Tyr- tyrosine, Val- valine.

#### 18 Extraction Procedure II

19 The following procedure illustrates one mode of  
20 selective precipitation.

21 A solution of sodium caseinate was digested with  
22 trypsin (50:1, casein:trypsin) for one hour at 37°C with the  
23 pH maintained at 8.0 by the addition of NaOH. HCl (0.1M) was  
24 then added to the solution at room temperature to pH 4.7 and  
25 the resulting precipitate removed.  $\text{BaCl}_2$  was added to the  
26 supernatant to a level of 0.25% w/v followed by an equal  
27 volume of absolute ethanol and the resulting precipitate was  
28 removed and dried. The precipitate was dissolved in one  
29 tenth the original volume of water (to facilitate  
30 dissolution the pH was raised with NaOH) and the solution  
31 acidified to pH 3.5 with 1M HCl. An equal volume of acetone  
32 was added and the precipitate removed and dried. The  
33 precipitate was then redissolved in  $\text{H}_2\text{O}$  and acidified to pH  
34 2.0 by addition of HCl. The resulting precipitate was  
35 removed and discarded and the supernatant was adjusted back  
36 to pH 3.5 with NaOH and an equal volume of acetone was  
37 added. The resulting precipitate was collected, redissolved  
38 in water and  $\text{H}_2\text{SO}_4$  added to precipitate  $\text{BaSO}_4$  which was

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1 discarded. The supernatant was then dialysed and  
2 lyophilised or spray dried. A mixture of 5 phosphopeptides  
3 were obtained with this procedure.

4 The following are the phosphopeptides obtained:-

5 T1.Glu-Met-Glu-Ala-Glu-Pse-Ile-Pse-Pse-Pse-Glu-Glu-Ile-Val-  
6 Pro-Asn-Pse-Val-Glu-Gln-Lys.

7 T2.Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu-Pse-  
8 Leu-Pse-Pse-Pse-Glu-Glu-Ser-Ile-Thr-Arg.

9 T3.Asn-Thr-Met-Glu-His-Val-Pse-Pse-Pse-Glu-Glu-Ser-Ile-Ile-  
10 Pse-Gln-Glu-Thr-Tyr-Lys.

11 T4.Asn-Ala-Asn-Glu-Glu-Glu-Tyr-Ser-Ile-Gly-Pse-Pse-Pse-Glu-  
12 Glu-Pse-Ala-Glu-Val-Ala-Thr-Glu-Glu-Val-Lys.

13 T5.Glu-Gln-Leu-Pse-Pth-Pse-Glu-Asn-Ser-Lys.

14 The ratio of the phosphopeptides (T1:T2:T3:T4:T5) in  
15 the final preparation depends on the starting material and  
16 conditions of hydrolysis. Digesting sodium caseinate with  
17 TPCK-trypsin yields largely T2 with small amounts of T1, T3  
18 and T4. However, T2 shows greater lability than the other  
19 peptides such that more rigorous digestion as occurs with  
20 some commercial casein digests yields a preparation  
21 containing largely T1 with small amounts of T3 and T4.

22 If in lieu of sodium caseinate, alpha s1-casein is used  
23 for this procedure pure T1 is obtained. With beta-casein as  
24 the starting material pure T2 is obtained.

25 The most common sequences of the active peptides is the  
26 pentapeptide Pse-Pse-Pse-Glu-Glu. The spacings of the  
27 phosphate and carboxyl groups in a beta-conformation of this  
28 pentapeptide are shown in Fig 1.

29 The 6.88 Angstrom spacings of phosphates and carboxyls  
30 allows specific attachment to calcium atoms along the c-axis  
31 of hydroxyapatite crystals. This pentapeptide sequence  
32 occurs in peptides T1 to T4 and occurs modified in peptide  
33 T5 - Pse-Pth-Pse-Glu-Glu following a conservative  
34 substitution of phosphothreonine for phosphoserine.

35 Conservative substitutions within the active sequence  
36 would be phosphothreonine and to a lesser extent  
37 phosphotryrosine or phosphohistidine for phosphoserine  
38 although phosphoserine is preferable. Another possible

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1 substitution for phosphoserine would be glutamate or  
2 aspartate but again phosphoserine is preferable. A possible  
3 substitution for glutamate is aspartate.

4 The active peptides can sequester calcium phosphate and  
5 other salts of divalent metal ions. One mole of T1 binds 16  
6 mole of  $\text{CaHPO}_4$  such that a 10mg/ml solution of T1 at pH 7.0  
7 can solubilize 60mM  $\text{CaHPO}_4$  producing a metastable  
8 supersaturated solution with respect to calcium phosphate  
9 species. With chloride as the counter ion one mole of T1  
10 binds only 5 mole of  $\text{Ca}^{++}$  binding only to serine phosphates.  
11 One mole of T1 with about 16 mole of  $\text{CaHPO}_4$  bound (M.W.  
12 4883) will henceforth be referred to as calcium phosphate  
13 T1. An important chemical feature of calcium phosphate T1  
14 is that above 2% w/v in water the composition is a  
15 thixotropic gel. T1-T5 have been shown to be potentially  
16 anticarcinogenic using the following test procedures:

17 Test 1. Hydroxyapatite Dissolution Rate Assay.

18 This test is a modification of a test procedure already  
19 described (Reynolds, E.C., Riley, P.F. and Storey, E.  
20 Calcif. Tiss Int 34:s52-s56, 1982). The purpose of this  
21 test is to determine the effect of the peptides on  
22 hydroxyapatite dissolution and in this respect since tooth  
23 enamel is largely composed of hydroxyapatite it is believed  
24 that useful comparisons can be made.

25 Double distilled, deionized water (18 mega ohms/cm) was  
26 used throughout. Analytical reagent grade chemicals were  
27 obtained from Ajax Chemicals, Australia. Hydroxyapatite-  
28 spherulodal was purchased from BDH. A chromatography column  
29 containing 0.1g of hydroxyapatite beads was used for the  
30 demineralisation assay. Tris 5mM, pH 8.3 containing 50mM  
31 NaCl was used as the column buffer at 20°C and was pumped  
32 through the column at a rate of 0.1ml/min. A peptide  
33 solution 0.1mg/ml of buffer was applied to the column and  
34 0.2ml fractions were collected before and after peptide  
35 application and assayed for total calcium, phosphate and  
36 peptide. From these values a rate of dissolution (nmol  
37 calcium or phosphate per min) for each 0.2ml fraction was  
38 obtained.

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1        Phosphopeptides T1-T5 all decreased hydroxyapatite  
2        dissolution rate by about 32%.

3        Phosphopeptides T6-T9 were found to be much less  
4        effective.

5        Fluoride plus phosphopeptide T1 gave a combined  
6        reduction in hydroxyapatite dissolution (40% reduction).  
7        The phosphopeptide T1 caused a 50% greater retention of  
8        fluoride in the hydroxyapatite column.

9        This work shows that these phosphopeptides bind to  
10       hydroxyapatite and reduce the minerals dissolution rate and  
11       enhance the retention of fluoride in the crystal matrix.  
12       The reduction in hydroxyapatite dissolution was related to  
13       the phosphoserine content and spacings within the peptides.

14       Test 2.    Intra-Oral Caries Test.

15       The anticariogenicity of phosphopeptide T1 was  
16       determined using a modification of the intra-oral caries  
17       test of Koulourides and Ostrom (Caries Res. 10:442-482,  
18       1978).    Enamel slabs were inset in a removable intra-oral  
19       appliance to simulate an approximal area.    This was done on  
20       both sides of the removable appliance (left and right).  
21       The appliance was worn to allow plaque accumulation in the  
22       simulated approximal areas.    Eight times a day the  
23       appliance was removed and placed in a solution at 37°C.  
24       The solution was 2% w/v sucrose, 2% w/v glucose, 140 mM KCl,  
25       20mM NaCl at pH 7.0.    Twice a day the right side enamel  
26       slabs received exposure to a solution containing 1.8% w/v  
27       calcium phosphate T1 in 140 mM KCl, 20 mM NaCl at pH 7.0,  
28       while the left side received only the salt solution.    At  
29       the completion of the experiment the enamel slabs were  
30       removed, sectioned and subjected to microradiography and  
31       microhardness testing.    The microradiography showed that  
32       the slabs exposed to the sugar-salt solution (left-side) had  
33       sub-surface, caries-like lesions.    However, the slabs  
34       exposed to the sugar-salt solution and the peptide T1  
35       solution twice a day showed no caries-like changes.    The  
36       results were confirmed by microhardness analysis.    Plaque  
37       was also taken from both sides of the appliance and analysed  
38       for calcium phosphate, serine phosphate and peptide T1 using

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1 a competitive, quantitative, enzyme-linked immunosorbent  
2 assay (ELISA) utilising monoclonal antipeptide T1  
3 antibodies.

4 This showed that the plaque on the right side of the  
5 appliance exposed twice a day to the peptide T1 solution  
6 contained the peptide at a level of at least 0.4% w/wet wt  
7 of plaque and the level of calcium phosphate had increased  
8 2-4 fold.

9 This work shows that peptide T1 is incorporated into  
10 plaque thereby increasing the plaque level of calcium and  
11 phosphate so inhibiting the caries process. This method of  
12 incorporation and accumulation in dental plaque can be used  
13 to carry remineralising and antibacterial ions into plaque  
14 and enamel e.g. Ca,  $PO_4$ ,  $FPO_3$ , Zn, Cu, Sn, Ag, Al, Fe and  
15 La.

16 Test 3 - Intra-Oral Remineralisation

17 An intra-oral appliance similar to that used in the  
18 previous test procedure was used except that the enamel  
19 slabs had been previously exposed to a demineralising  
20 solution to produce two sub-surface demineralised lesions in  
21 each slab. The demineralising solution was a 0.1M lactate  
22 buffer pH 5.0 containing 500 mg/L hydroxyapatite and 1%  
23 agar. The appliances were worn by subjects for 10 days.  
24 Twice each day the appliances were removed and a drop of  
25 remineralising solution was placed on the enamel slabs on  
26 the right of the appliance. The left-side enamel slabs  
27 served as controls. After 10 days the enamel slabs were  
28 removed, sectioned and subjected to microradiography. The  
29 amount of mineral deposited back into the sub-surface  
30 lesions was determined using microdensitometry. The  
31 remineralising solution containing 1.8% w/v calcium  
32 phosphate T1 pH 7.0 returned 57% of the mineral lost  
33 compared with 13% by saliva alone.

34 Test 4 - Plaque pH Fall

35 Subjects refrained from oral hygiene for 3-5 days then  
36 rinsed with a 5% sucrose solution for 1 min. Plaque samples  
37 were removed and pH was measured using the one drop  
38 technique. After approximately 5 min the pH fell to around

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1 5.0. However, if the subjects rinsed with a solution  
2 containing 1.8% w/v calcium phosphate T1, pH 7.0 15 min  
3 before rinsing with the 5% sucrose solution the plaque pH  
4 did not fall below 6.7, demonstrating significant pH  
5 buffering by the calcium phosphate T1.

6 While the precise mechanism by which the  
7 phosphopeptides exhibit anticariogenic activity is not  
8 known, the following speculative theories have been put  
9 forward but are not to be taken as binding or limiting.

10 The phosphopeptides may accumulate in plaque and  
11 enamel, buffer plaque acid, prevent enamel demineralisation  
12 and enhance remineralisation. The small molecular weight  
13 of the phosphopeptides may allow penetration and  
14 accumulation in plaque and enamel pores. The  
15 phosphopeptides, due to the appropriate spacing of serine  
16 phosphate residues, may bind to tooth enamel mineral and  
17 prevent demineralisation. The peptides may also carry  
18 calcium and phosphate (fluorophosphate on modification) into  
19 plaque and enamel, in an appropriate form, possibly allowing  
20 spontaneous remineralisation. The phosphoserine residues  
21 may also buffer plaque acid. The phosphopeptide may also  
22 carry antibacterial metal ions e.g. Zn, Cu, Sn, Ag, Al, Fe  
23 and La into plaque and in this way have an antiplaque and  
24 antigingivitis effect. The metal ions are carried by the  
25 phosphopeptides primarily due to the phosphoserine residues.  
26 Phosphopeptides may bind to plaque bacteria and inhibit  
27 sugar utilisation.

28 The ability of these peptides to sequester calcium  
29 phosphate can be utilised in the treatment of various  
30 rarefying bone diseases. These peptides can significantly  
31 increase the absorption of calcium, phosphate and iron in  
32 the gut. Hence, pharmaceutical vehicles (e.g. enteric  
33 capsules) or foods containing calcium phosphate T1 and  
34 ferrous phosphate T1 can be used for the treatment of  
35 osteoporosis/osteomalacia and anaemia.

36 Applicants have formulated various compositions in  
37 accordance with this invention as follows. In general, the  
38 compositions contain from 0.01-10% by weight of

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1 phosphopeptide.

2 Example 1. Flour: In a device for mixing dry  
3 substances, 1% by weight of calcium phosphate T1 was blended  
4 with flour.

5 Example 2. Cereal: A breakfast cereal was sprayed with  
6 a solution of calcium phosphate T1 in water. The cereal  
7 flakes were then dried to produce a finished product  
8 containing 1% calcium phosphate T1.

9 Example 3. Bread: 1% by weight of calcium  
10 phosphate T1 was added to the flour during the mixing of  
11 ingredients for the manufacture of bread.

12 Example 4. Cake mix: 1% by weight of calcium  
13 phosphate T1 was added to the dry ingredients in the  
14 preparation of a cake mix.

15 Example 5. Confectionery: In the preparation of  
16 confectionery 1% by weight of calcium phosphate T1 was added  
17 to the final mixture.

18 Example 6. Biscuit: In the preparation of a  
19 biscuit/mixture 1% by weight of calcium phosphate T1 was  
20 added to the other dry ingredients during mixing.

21 Example 7. Beverage: A beverage was prepared in which  
22 0.1% weight of calcium phosphate T1 had been dissolved.

23 Example 8. Tablet: A tablet was made containing 10% by  
24 weight of calcium phosphate T1 together with excipients  
25 being flavouring matter and binding material.

26 In preparation of a typical dentifrice within the scope  
27 of this invention, the requisite salt and salts of the  
28 selected phosphopeptide are incorporated into dentifrice  
29 compositions in any suitable manner depending on whether a  
30 powder, paste or liquid preparation is to be produced. For  
31 this purpose appropriate preparations of surface-active  
32 agents, binders, flavouring materials and other excipients  
33 required to achieve the required form of dentifrice are  
34 added.

35 The invention is further illustrated by the following  
36 examples:

37 Example 9. Tooth paste: A toothpaste was prepared  
38 having the following composition:

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1	Calcium phosphate T1	5.0% by weight
2	CMC 7MF	1.0% " "
3	Saccharin 450	0.2% " "
4	Glycerin (B.P.)	25.0% " "
5	Sodium lauryl sulphate	
6	(Empicol 0919)	5.0% " "
7	Sodium benzoate	0.5% " "
8	Flavour 9/693090	0.8% " "
9	Calcium phosphate	1.0% " "
10	Water Deionised	39.5% " "
11	Thixosyl 33J	9.5% " "
12	Syloid AL-1	12.0% " "
13	Titanium Dioxide 3328	0.5% " "

14 Example 10. Toothpaste: A preparation as set out in  
 15 Example 9 was repeated but with the addition of 0.2% sodium  
 16 fluoride in a suitable form.

17 Example 11. Toothpaste: A preparation as set out in  
 18 Example 9 was repeated but with the addition of 0.4%  
 19 stannous fluoride in a suitable form.

20 Example 12. Toothpaste: A preparation as set out in  
 21 Example 9 was repeated but with the addition of 0.76%  
 22 monosodium fluorophosphate in a suitable form.

23 Example 13. Toothpowder: The following preparation was  
 24 made:

25	Calcium phosphate T1	5.0% by weight
26	Soluble saccharin	0.1% " "
27	Colour agent	Trace
28	Dibasic calcium phosphate	94.1% " "

29 Example 14. Toothpowder: A preparation as set out in  
 30 Example 13 was made but with the addition of 0.78%  
 31 monosodium fluorophosphate in a suitable form.

32 Example 15. Liquid dentifrice: A preparation was made  
 33 consisting of:

34	Sodium alginate	1.4% by weight
35	Calcium phosphate T1	2.0% " "
36	Sodium lauryl sulphate	1.0% " "
37	Flavouring	Trace
38	Colouring	Trace



1           Water                                 95.5% " "

2 Example 16.      Liquid dentifrice: As for Example 15 but

3 with 0.5% sodium fluoride added.

4 Example 17. Mouthwash: The following preparation was  
5 made:

6	Calcium phosphate T1	2.0% by weight
7	Sodium fluoride	0.5% " "
8	Flavouring	Trace
9	Colouring	Trace

10	Water	97.5%	"	"
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11 Example 18. Carbonated beverage: 0.1% by weight of  
12 calcium phosphopeptide T1 was added to a commercially  
13 available carbonated beverage.

14 Example 19. Fruit juices: 0.1% by weight of calcium  
15 phosphopeptide T1 was added to a commercially available  
16 fruit juice.

17 Example 20. Solution for topical application to teeth.

18	Calcium Phosphate T1	2%
19	NaF	0.8 mM
20	ZnAcetate	0.1 mM
21	SrCl <sub>2</sub>	0.1 mM

22 (this solution may be formed into gel by increasing the  
23 amount of calcium phosphate T1).

24 Example 21. Dental filling material

25	Calcium phosphate T1	5% w/w
26	Calcium phosphate	95% w/w
27	Polymeriser	trace

28      Made as a paste with water

29       The polymeriser used in this example was  
30   glutaraldehyde.

31 Example 22. Dental filling material.

32	Calcium phosphate T1	5% w/w
33	Calcium phosphate	70%
34	Acrylic polymer	25%
35	Catalyst for polymer	trace

36 Example 23. Topical Gel for the Treatment of hypersensitive  
37 teeth.

38	Calcium phosphate T1	4.0% by weight
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1	SrF <sub>2</sub>	1.0% by weight
2	Flavouring	Trace
3	Water	95%

4 In the above calcium phosphate T1 was used for illustration  
5 but in lieu any appropriate phosphopeptide and/or salt might  
6 be used.  
7 Modifications and adaptations may be made to the above  
8 described without departing from the spirit and scope of  
9 this invention which includes every novel feature and  
10 combination of features disclosed herein.  
11 The claims form part of the disclosure of this

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1 THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

- 2 1. A phosphopeptide or a salt thereof the phosphopeptide  
3 having from 5 to 30 amino acids including the sequence  
4 A-B-C-D-E  
5 where A,B,C,D and E are independently phosphoserine,  
6 phosphothreonine, phosphotyrosine, phosphohistidine,  
7 glutamate and aspartate.
- 8 2. A phosphopeptide as claimed in claim 1, wherein A,B and  
9 C are independently phosphoserine, phosphothreonine,  
10 phosphotyrosine and phosphohistidine and D and E are  
11 independently phosphoserine, phosphothreonine, glutamate and  
12 aspartate.
- 13 3. A phosphopeptide as claimed in claim 1, where A,B and C  
14 are phosphoserine and D and E are glutamate.
- 15 4. A phosphopeptide being one of Glu-Met-Glu-Ala-Glu-Pse-  
16 Ile-Pse-Pse-Pse-Glu-Glu-Ile-Val-Pro-Asn-Pse-Val-Glu-Gln-Lys.
- 17 5. A phosphopeptide being one of Glu-Leu-Glu-Glu-Leu-Asn-  
18 Val-Pro-Gly-Glu-Ile-Val-Glu-Pse-Leu-Pse-Pse-Pse-Glu-Glu-Ser-  
19 Ile-Thr-Arg.
- 20 6. A phosphopeptide being one of Asn-Thr-Met-Glu-His-Val-  
21 Pse-Pse-Pse-Glu-Glu-Ser-Ile-Ile-Pse-Gln-Glu-Thr-Tyr-Lys.
- 22 7. A phosphopeptide being one of Asn-Ala-Asn-Glu-Glu-Glu-  
23 Tyr-Ser-Ile-Gly-Pse-Pse-Pse-Glu-Glu-Pse-Ala-Glu-Val-Ala-Thr-  
24 Glu-Glu-Val-Lys.
- 25 8. A phosphopeptide bein one of Glu-Gln-Leu-Pse-Pth-Pse-  
26 Glu-Glu-Asn-Ser-Lys.
- 27 9. A phosphopeptide or a salt thereof as claimed in any  
28 preceding claim and in substantially pure form.
- 29 10. A mixture of phosphopeptides or salts thereof wherein a  
30 phosphopeptide or salt thereof in accordance with any one of  
31 claims 1-9 predominates.
- 32 11. A composition comprising a phosphopeptide or a salt  
33 thereof in accordance with any one of claims 1-9 together  
34 with a physiologically acceptable diluent.
- 35 12. A composition as claimed in claim 11, wherein the  
36 phosphopeptide or salt thereof is present in the composition  
37 as 0.01 to 10% by weight.
- 38 13. A composition as claimed in claim 11, wherein the

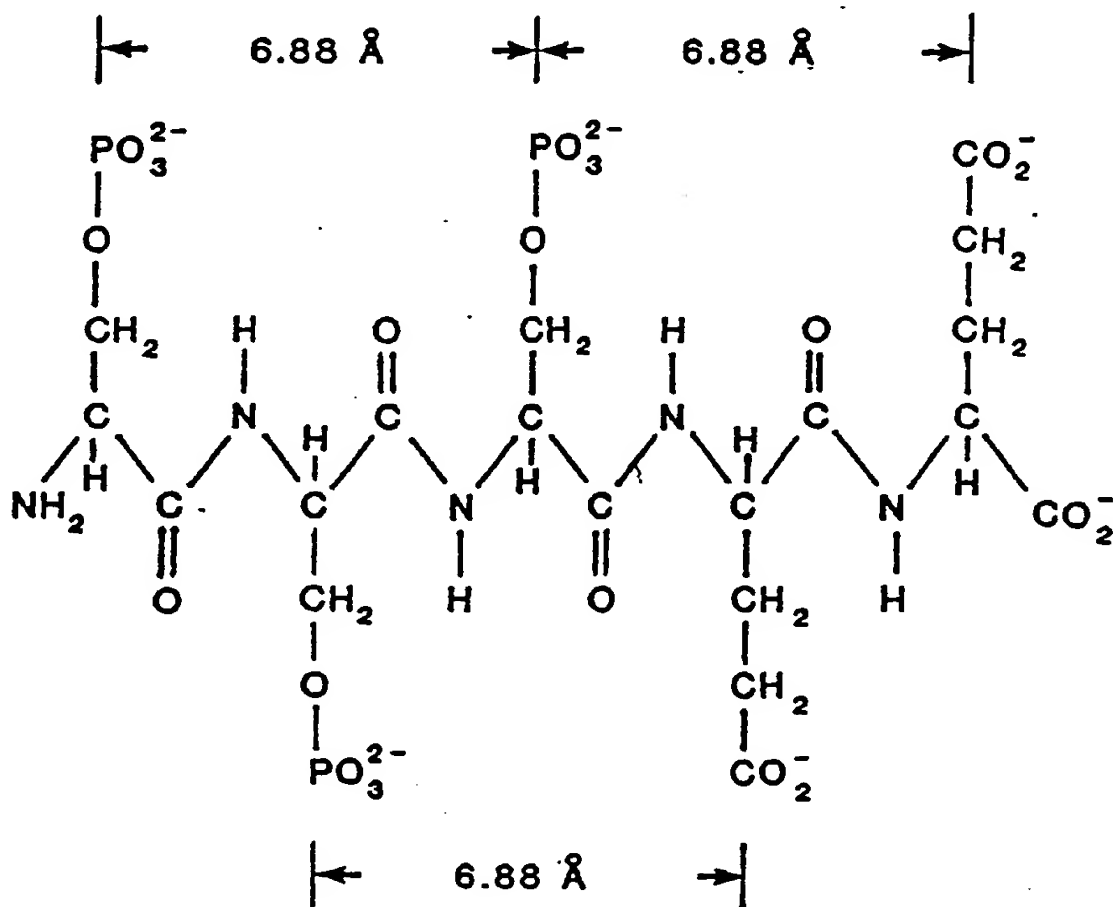
- 18 -

- 1 phosphopeptide or salt thereof is present in the composition  
2 as 0.01 to 5% by weight.
- 3 14. A composition as claimed in claim 11, wherein the  
4 phosphopeptide or salt thereof is present in the composition  
5 as 0.01 to 2% by weight.
- 6 15. A composition as claimed in any one of claims 11-14,  
7 wherein the diluent is a pharmaceutically acceptable  
8 diluent.
- 9 16. A composition as claimed in any one of claims 11-14,  
10 wherein the diluent is an orally ingestible material.
- 11 17. A composition as claimed in claim 16, wherein the  
12 diluent is a comestible.
- 13 18. A composition as claimed in claim 17, in the form of a  
14 foodstuff or confection.
- 15 19. A composition as claimed in any one of claims 11-14, in  
16 the form of a toothpaste, tooth powder, dentifrice,  
17 mouthwash or preparation for topical application to teeth or  
18 gingival tissue.
- 19 20. A composition as claimed in any one of claims 11-14, in  
20 the form of a gel.
- 21 21. A composition as claimed in any one of claims 11-14, in  
22 the form of a dental filling composition.
- 23 22. A composition as claimed in claim 21 and additionally  
24 containing calcium phosphate or hydroxyapatite.
- 25 23. A method of obtaining a phosphopeptide in accordance  
26 with any one of claims 1-9 which comprises fractionating a  
27 digest of casein, alpha-s-casein, beta-casein or a salt  
28 thereof.
- 29 24. A phosphopeptide in accordance with anyone of claims 1-  
30 9 in combination with calcium phosphate or hydroxy apatite.
- 31 25. A combination in accordance with claim 24, comprising  
32 about 16 mole of  $\text{CaHPO}_4$  per mole of phosphopeptide.
- 33 26. A combination in accordance with claim 24, or claim 25  
34 in the form of a solution or gel.
- 35 27. A phosphopeptide or salt thereof, composition  
36 containing same or a method of obtaining same substantially  
37 as hereinbefore described with reference to any one of the  
38 Examples.

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1 28 The articles, things, parts, elements, steps, features,  
2 methods, processes, compounds and compositions referred to  
3 or indicated in the specification and/or claims of the  
4 application individually or collectively, and any and all  
5 combinations of any two or more of such.

Fig. 1



## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE <sup>1</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ..... because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claim numbers 28, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

The claim is indefinite

3. ☐ Claim numbers ..... because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING <sup>2</sup>

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International Application No PCT/AU 87/00172

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int. Cl <sup>4</sup> C07K 7/06, 7/08, 7/10, 15/12, A61K 37/16, 37/18, 7/26, C12P 21/06, A23J 1/20, 1/12, 1/14		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched *		
Classification System	Classification Symbols	
IPC	C07K 7/06, 7/08, 7/10, C07C 103/52	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched *		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT *</b>		
Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages **	Relevant to Claim No. **
A	DE,A, 3320175 (BOEHRINGER MANNHEIM GmbH) 6 December 1984 (06.12.84)	(1-27)
A	US,A, 4358465 (G BRULE) 9 November 1982 (09.11.82)	(1-27)
A	US,A, 4361587 (G BRULE) 30 November 1982 (30.11.82)	(1-27)
A	US,A, 4495176 (G BRULE) 22 January 1985 (22.01.85)	(1-27)
A	AU,A, 19567/83 (ELI LILLY AND COMPANY) 5 April 1984 (05.04.84)	(1-27)
A	AU,B, 39619/78 (520417) (SANDOZ LTD) 13 March 1980 (13.03.80)	(1-27)
<p>* Special categories of cited documents: **</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"G" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
20 August 1987 (20.08.87)	01 September 1987 (0-09-87)	
International Searching Authority Australian Patent Office	Signature of Authorized Officer R. SAWYER	



ANNEX TO THE INTERNATIONAL SEARCH REPORT ON  
INTERNATIONAL APPLICATION NO. PCT/AU 87/00172

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

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END OF ANNEX

